Comunicado Técnico 129

ISSN 1678-1937
November, 2013
Aracaju, SE

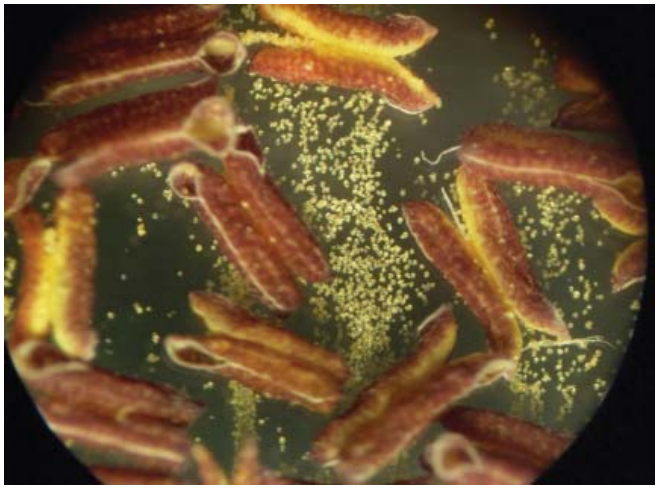

Photo: Adriane Leite do Amaral

Polen Storage Metodology in Sugarcane

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The flowering synchronization of genitor plants is fundamental to perform sugarcane crossings. Research on flowering induction has always been among the main interests of sugarcane breeding programs. Synchronized planting and artificial light supplements have been employed without good efficiency.

The conservation of sugarcane pollen can be used as an auxiliary methodology for asynchronous crossing. Many methodologies have already been tested but have neither been confirmed nor given efficient results (KRISHNAMURTHI, 1977; MOORE; NUSS, 1987; YONGHUI et al., 1993; YAOHUI; OIYAO, 1994; PRASAD).

For these reasons, sugarcane breeding researchers have not yet settled on a pollen conservation methodology to be used as a standard asynchronous crossing.

The methodology described in this publication was a result of a series of studies on pollen conservation that have been developed since 2009 in partnership with the Brazilian Agricultural Research Corporation (Embrapa) and the Interuniversity Network for the Development of the Sugar-Energy Industry (Ridesa).

The studies were conducted at the Serra do Ouro Flowering and Crossing Breeding Station in the city of Murici, AL (09º 13'S, 35º 50'W), at an altitude of 450 m above sea level and 34 km away from the coast, and in the Research and Development Unit (UEP) laboratory, Embrapa Coastal Tablelands in the city of Rio Largo, which is located inside the Center for Agricultural Science of the Federal University of Alagoas (UFAL), (09º 28'S, 35º 47'W, 141 m altitude above sea level).

At first, the preservation method was composed of temperature and moisture content variations in storage (AMARAL et al., 2011). Secondly, it was adapted to pollen viability techniques and assessments, aiming to monitor the pollen conservation (AMARAL et al., 2013). Finally, several crossings with stored pollen were done and the plants were analyzed with molecular markers for confirming the crossings in the proposed methodology (AMARAL et al., 2012).

This publication aims to describe the method of pollen preservation and viability assessment in sugarcane. This method was developed in partnership with Embrapa and Ridesa in order to facilitate the crossings between asynchronous flowering varieties of sugarcane.

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Methodology

Before collecting the pollen

For the collection of sugarcane pollen, it is necessary to identify flowering and pollen viability aspects before the storage, such as:

Botany

The sugarcane inflorescence is a panicle with thousands of sessile spikelets. Each spikelet is a hermaphrodite flower, because both sexes are present in the same flower, androecium (male) and pistil (female). In the gynoecium or pistil, stigma can vary in color from red to purple, which characterizes the appearance of the purple panicles. The androecium has three stamens and each stamen has one anther (Blackburn, 1984) (Figures 1A, 1B and 1C). Normally, the pistil is receptive just prior to the androecium in one flower featuring protogyny phenomenon and favoring the cross-fertilization.

The panicle has a gradual maturation, which happens from the apex to the base, so that the apex flowers mature before the flowers in the base of the panicle. Thus, the dehiscence and pollen release can be gradually observed in every 1/3 of the inflorescence.

Photos: Adriane Leite do Amaral

Figure 1. Details of the sugarcane inflorescence: sessile spikelets (A), gynoecium with purplish and fluffy stigmas (B), and anthers in plain pollen dehiscence (C).

Sexing

Sugarcane flowers are generally considered hermaphroditic. Some varieties can behave like female flowers, when they do not have any anthers, or the anthers do not have pollen, or the pollen is unviable; and some behave like male flowers, when there is viable pollen production in the flowers. For the conservation of pollen, it is important to select varieties with fertile male flowers or hermaphroditic flowers, so the female flowers are discarded. Sexing should be done annually as part of the Germplasm Bank characterization and must preceed the artificial sugarcane crossing.

The sugarcane sexing practice is usually done with cytological staining and stereomicroscope visualization. This practice is also limited to pollen features and ignores the fertility and egg sack and embryo conditions. For practical purposes, plants with more than 70% of fertile pollen grains are considered male genitors in artificial crossings, and those with less than 30% as female genitors (GÓMEZ, 1962) (Figures 2A, 2B and 2C).

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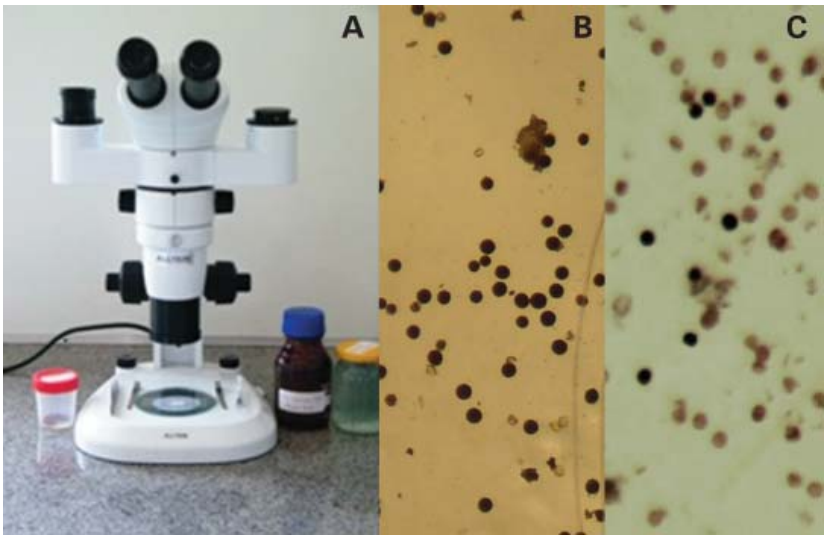

Figure 2. Sugarcane sexing: pollen analyses in a stereoscopic microscope (A), to visualize the viable (stained) and nonviable (unstained) pollen grains in Lugol solution, and to classify the varieties to use in crossings with male (B) or female (C) genitors according to the viable pollen proportion.

Pollen collection

The sugarcane flowers in different regions of Brazil. This happens especially in the Northeast coastline of Brazil, latitudes between 0° to 15°S, where there is a high favorability for the collection and preservation of sugarcane pollen because of the high pollen viability.

An ideal place for flowering and pollen collection needs to have inductive conditions like photoperiod from 12 to 12.5 hours per day (ARCENEAUX 1967; CLEMENTS, AWADA, 1967), latitudes between 10ºN and 10ºS, (CESNIK; MIOQUE, 2004), temperature in the flowering induction phase should be greater than 18ºC and below 31ºC (PEREIRA et al., 1983) and air humidity above 60%, which are ideal for flowering and pollen viability. In the absence of any of these inductive conditions, flowering becomes occasional or may not even occur. Small variations in air temperature can cause major changes in the flowering and pollen fertility. Temperatures lower than 18ºC are harmful to the flowering. CASTRO (2001) experienced losses in the induction of flowering when there were temperatures lower than 18ºC after five consecutive nights. ELLIS et al., (1967) and LEVI (1983) demonstrated that high temperatures exceeding 32-35°C are also considered harmful to the sugarcane flourishing

Time of pollen collection

The sugarcane flowering occurs at different times in Brazil ranging from March to September. The flowering depends on the latitude; at the lower latitudes it begins in March and at the higher latitudes, it begins in September. In addition to the equatorial regions (latitudes close to zero), the flowering can be induced at any time of the year. In the state of Alagoas, Brazil (09º 13'S, 35º 50'W), the flowering period is concentrated from April to July (about 90 days / year), which is the preferential time to collect the sugarcane pollen. RIDESA's sugarcane breeding program classifies the varieties in early, intermediate or late flowering, according to the time of the spontaneous flowering. This flowering time classification is important because it defines the crossings that could be planned out. Varieties with early and late flowering can not be intercrossed due to the temporal barrier on the development of flowers. Sugarcane varieties with intermediate flowers can be crossed with early or late flowers, since techniques to promote the floral synchrony should be used.

For the methodology establishment purpose, the early varieties are the ones which spontaneously flower in April, the intermediate varieties which flower in May and the late flowers in June were all considered.

Pollen collecting

Prior to the collection, the pollen should be considered to be extremely susceptible to dehydration. With a half-life of only 12 minutes, the pollen grains do not survive more than 35 minutes in an environment at 26.5°C and a relative humidity of 67% (VENKATRAMAN, 1922 MOORE, 1976, AUSTRALIA, 2004, 2008). To collect pollen, the inflorescences must be harvested in the morning, preferably between 7 and 8 a.m. to prevent dehydration by sun exposure and high temperatures. For the pollen collection, the inflorescences should be chosen when the anthers are ripe and releasing pollen naturally in at least one third of the panicle.

In the field, the inflorescence stems should be cut with a minimum length of 100 cm and immediately sent to the laboratory (Figures 3A, 3B and 3D). For transporting from

the field to the lab, the panicles should be individually placed in paper bags and packed in styrofoam boxes with ice. It is recommended that the cutting and arrival of the panicles at the laboratory must have a maximum time interval of thirty minutes.

If the flowering and pollen collection happen on rainy days, it is recommended to collect the inflorescences (with pollen dehiscence in the first third of the panicle) even though they are wet, and then preserve them in Mangelsdorf's nutrient solution (CESNICK; MIOCQUE, 2004). To promote anthesis in the central or/and basal portion of the panicles, they should be kept in Mangelsdorf's nutrient solution for a period of approximately 24 hours. This procedure makes it possible to collect pollen immediately after the anthesis (Figures 3C and 3E).

For the inflorescence, it is recommended that the laboratory temperature be approximately 23 ± 1°C so the panicles can be scrubbed with brushes on a smooth surface with uniform color, which favors the extraction and visualization of the pollen (Figures 3F and 3G). The collected pollen and anthers should then be stored in glass or plastic vials, in which the top and bottom should have the same diameter and screw on caps, filled to the maximum volume of 1/4. The amount of pollen from an extracted anther is variable; however, thirty panicles can provide 1000-2000 mg of pollen.

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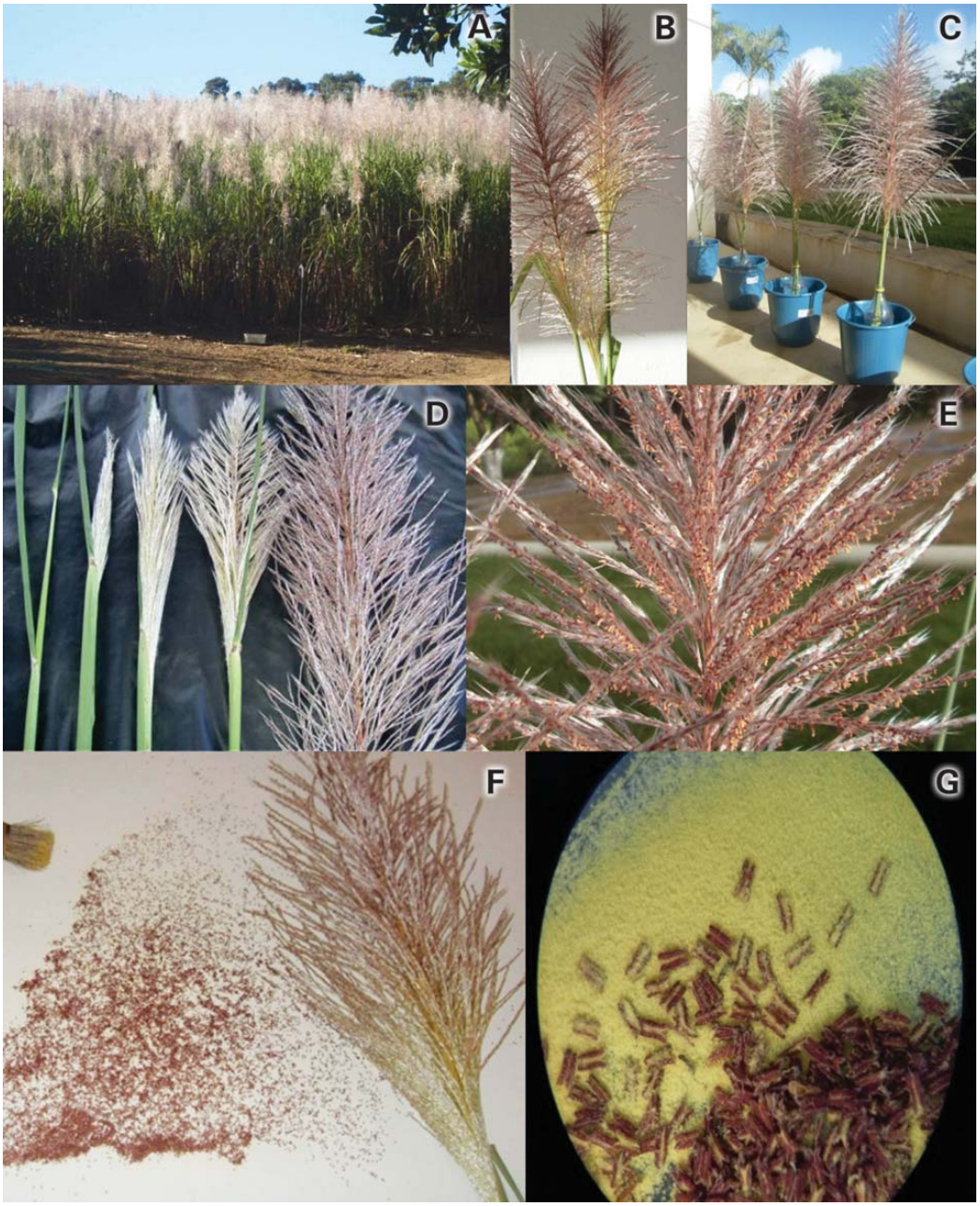

Figure 3. Sugarcane inflorescences and pollen harvesting: a field with profuse and spontaneous sugarcane flowering (A), panicle collected (B) and conserved in nutritive solution for the harvesting pollen (C), panicle development (D), panicle with production and exposition of anthers and average portion (E), harvesting of pollen with brushes (F), and a magnified picture of pollen with harvested anthers (G).

Pollen dehydration in the pre-storage

APrior to the pollen conservation, which needs low temperature, some dehydration process must be used. The excess of moisture can generate ice crystals and damage tissues in low temperature storage (YOGEESHA et al., 1999; PRASAD, 2012). For dehydration in the pre-storage, blue silica gel is recommended, which has an adsorption capacity of up to 30% of its weight in water (HAIPENG et al. 1993). For dehydration of 2000 mg of pollen, it is advisable to divide this amount into four vials with 50 ml in each. The vials should be kept uncovered and placed in sealed glass desiccators containing 1000 g of blue silica gel. Each glass desiccator should include pollen from an individual variety to avoid contamination and the vials must be open for the silica to adsorb the moisture from the air and the pollen samples. The dehydration should occur for about one hour in a refrigerator (4°C) (Figure 4). It is possible to obtain an average reduction of 20% of the total sample weight through the dehydration process, which is important for reducing the moisture and promoting the pollen conservation (AMARAL et al. 2011).

Photos: Adriane Leite do Amaral

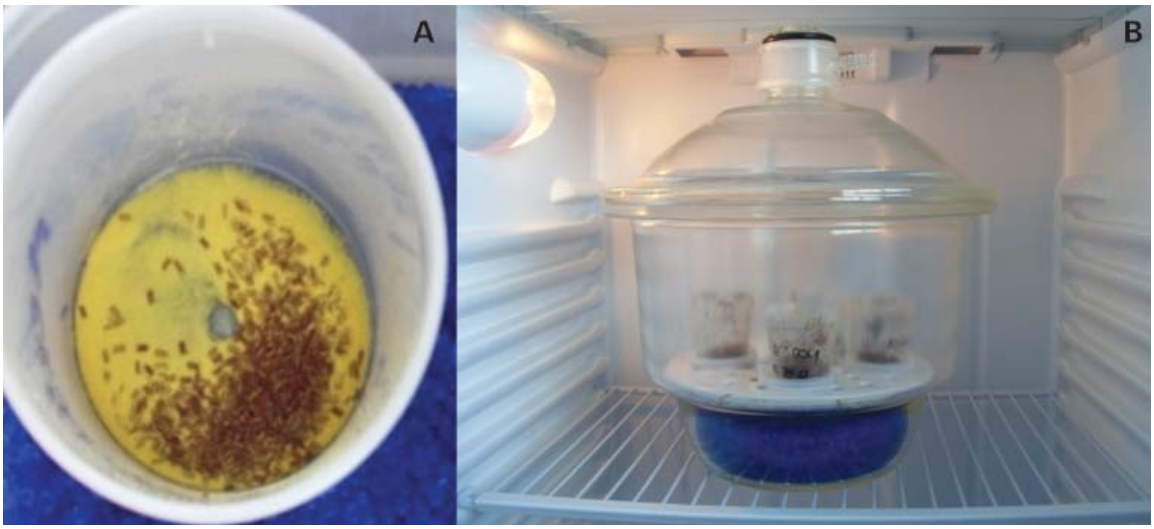

Figure 4. Dehydrated samples of sugarcane pollen in blue silica gel (A) in glass desiccators at 5°C (B).

Pollen storage

Immediately after the pollen dehydration, the vials must be covered, transferred to a freezer and then stored at -18° C (Figure 5). The results show that the samples containing pollen and anthers dehydrated in 20% of their weight and stored at -18°C can then be storage for more than thirty days (AMARAL et al. 2011; YAOHUI and OIYAO, 1994).

The thirty days of storage may be considered sufficient for getting synchrony between the varieties in bloom at different moments within the same flowering season.

However, there is the possibility of extending the pollen's shelf life to allow crossings between plants which flower in different flowering seasons.

The success in extending the pollen's shelf life and increasing the storage time depends on not only the collection and conservation of pollen technique, but also on the local environmental conditions and germoplasm which produce pollen.

Photo: Adriane Leite do Amaral

Figure 5. Vials with pollen samples stored in a freezer at -18°C.

Analyses of pollen viability

Pollen viability should be evaluated preferably by using different techniques and at least two moments, pre and post storage. It is important that the pollen viability evaluation be done immediately after the collection of pollen and immediately before the crossing (with thirty days of storage) to ensure the storage and crossing with viable pollen grains.

The pollen viability analysis is important in the storage process because when the initial feasibility of pollen is higher, the chances of obtaining plants from crossings with stored pollen will be greater

Samples of pollen grains with initial viability higher than 50% are recommended for the application of the sugarcane pollen storage method. The application of three simultaneous analyszes are recommended for an accurate assessment of the pollen viability:

i) Cytological integrity (staining with Lugol solution).

ii) In vitro viability (pollen germination in culture medium).

iii) In vivo viability (artificial crossings).

Cytological integrity

The cytological integrity analysis gives a preview of the quality of the collected pollen because it allows checking if there is a rupture in the membranes and/or deficiency in the starch reserves.

This analysis is performed by cytological staining technique, which uses Lugol's solution (1 g of iodine, 2g of potassium iodide and 100 ml of distilled water), which is the most used in sugarcane breeding programs (JR MACHADO, 1987).

For the staining, a small sample of pollen must be placed on a glass slide in a drop of Lugol solution. The pollen sample needs to be analyzed under a stereomicroscope (40X magnification). The pollen visualization and analysis can be done in different ways, for example: through the direct visualization of an experienced technician and through a digital camera attached to the microscope to capture the image, which can be viewed by human eye and/or by computer programs for image analysis.

For the cytological integrity evaluation, each slide shows a view, which is divided into four parts, corresponding to four replicates (Figure 6). Lugol solution staining allows to distinguish the pollen grain viability: i) pollen grains stained (variations of brown) suggest pollen viability because there is starch and intact membranes, and ii) discolored pollen grain (light yellow and/or translucent), indicating unviable pollen or poorly formed, because of the absence or deficiency of starch, membrane defects and/or ruptures (Figure 6A).

The counting of pollen stained and unstained grains allows to estimate the pollen viability percentage using the formula:

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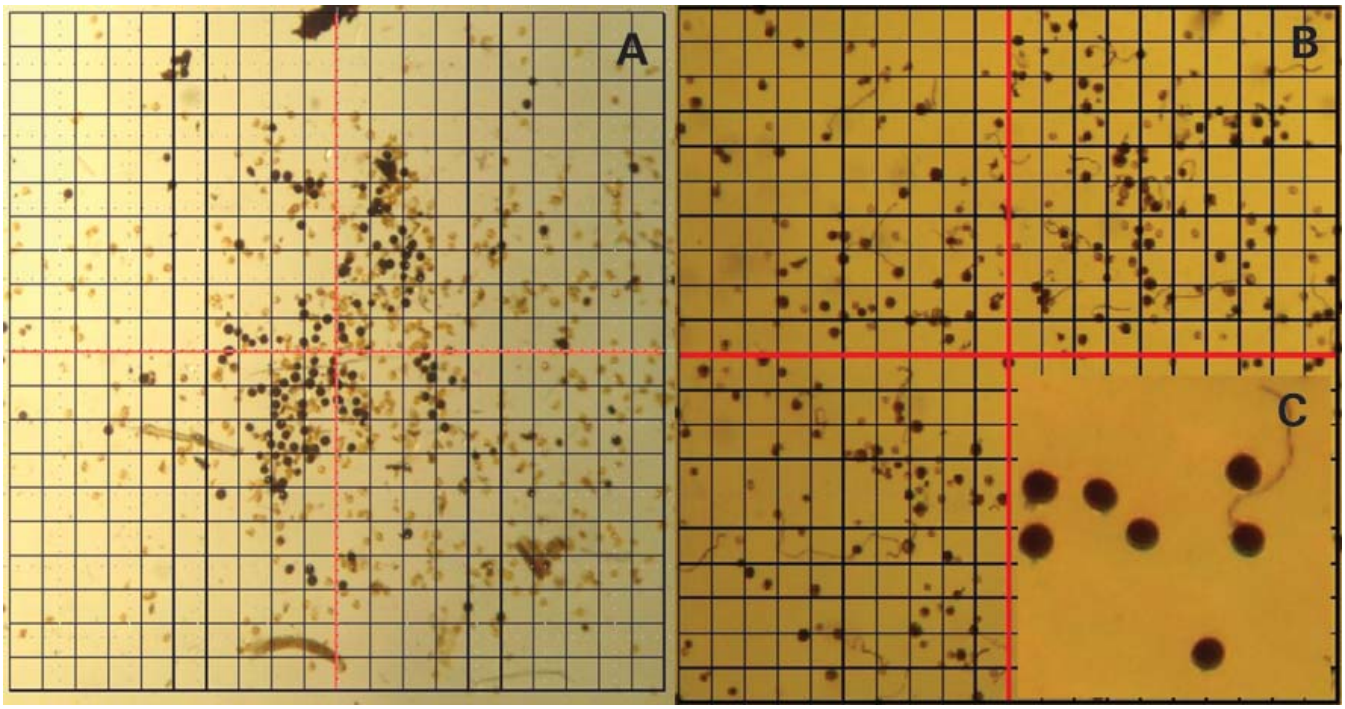

Figure 6. Pollen viability evaluation: cell integrity (A) and in vitro germination (B), pollinic tube in development (C).

In vitro viability

The culture medium adapted from KRISHNAMURTHI (1977) can be used for the evaluation of the pollen germination, which contains in its composition: Boric acid (100 ppm), calcium nitrate (60 ppm), magnesium sulfate (100 ppm) and 30% of sugar per 1 liter of H2O (AMARAL et al. 2013). This culture medium achieves better results than others suggested by YAOHUI and OIYAO (1994). The germination of pollen should be considered when the pollen tube length is exceeding the diameter of the pollen itself (WANG et al. 2004).

The following steps should be performed to assess the in vitro germination:

i) Place a pollen sample in a drop of culture medium on a histological slide.

ii) Allow the sample to rest for about an hour, leaving enough time for viable pollen grains to emit pollen tubes.

iii) Observe the slides under a stereomicroscope (40X magnification).

iv) Count and calculate the percentage of the germinated pollen.

The pollen germination should be measured at two different times: pre-storage (immediately after panicles harvest) and post-storage (30 days after pollen storage) (Figures 6B 6C).

In vivo viability

The in vivo pollen viability can be indirectly measured through the number of seeds and/or plants from crossings which are made with stored pollen.

Four steps are recommended for the assessment of the in vivo viability:

Female plant identification, collection and processing

Plants with visible flowering can be used as female parents (panicles with receptive stigmas, in at least one third of their lengths). Plants should be cut at the base of their stem and a part of the stems needs a to be immediately put inside of Mangelsdorf's solution and taken to a covered place where they will be processed. It is recommended to remove most of the leaves, to avoid excessive water loss (Figures 7A and 7B). For the emasculation, the panicles can be immersed in warm water (50 º C) for about 5 minutes (MACHADO JR et al., 1989). The emasculation process avoids pollen competition between the female plant and the stored pollen (Figure 7C).

Crossings

The crossings are done with stored pollen. The vails, containing the male stored pollen, must be removed from the freezer and kept in the shade and at room temperature (24º C) for fifteen minutes to defrost just before panicle pollenation of the female parents. Pollenation can be made with a brush or pouring the pollen in paper bags with the panicles (Figure 7D). Immediately after the pollination, panicles should be covered with hoods and maintained at rest in a cool area. The forest have high humidity and warm temperatures which favor the fertilization process (Figures 7E, 7F and FG).

It is recommended to use the pollen for two pollenations in sequential days with two different samples. After five days, the fertilized plants should be moved to a protected environment where they are left to rest for about thirty days for the seeds to develop and mature. During the seed maturation period, the nutrient solution must be replaced daily to prevent the plants from drying. After the development and maturation of the seeds, the panicles should be placed in a drying room, where the temperature (34 º C) and relative humidity need to be controlled (RH = 55 %). For the drying process, the panicles should be put inside paper bags which must remain open and aired during the period of 12 - 24 hours. After the drying process, the seeds must be manually separated from the rachis and stored in an environment with a temperature of 21º C and 60% of RH (SHINE et al., 1981). Finally, the delinting should be done, which consists of manually separating the seeds from the inert material (straw, stigma and others). The delinting needs to be done to avoid harming the sowing and germination of the seedlings caused by inert material (MARTINS, 2005).

Photos: Adriane Leite do Amaral

Figure 7. Sugarcane crossings at Serra do Ouro, Murici, AL, using 30 day stored pollen. Harvest of the female genitor panicles (A), removal of the leaves (B), emasculation (C), pollinization with stored pollen (D-E), panicles protected in hoods (F) and located beside a forest.

Sowing seeds

The sample of seeds (2 g) produced from crossings with stored pollen can be cultivated in boxes with substrate (two parts with fine sand and one part with organic soil). The germination of the seeds can be evaluated on the 20th day after being sowed (Figure 8). The seedlings with a minimum of 5 to 6 cm in height can be individually transplanted into 200 ml pots with substrate.

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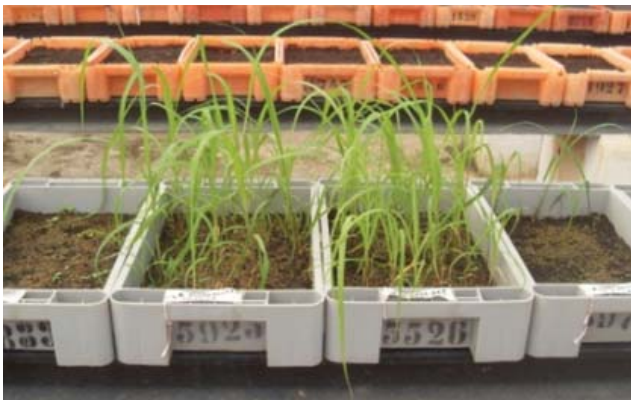

Figure 8. Plants generated in artificial crossings with 30 day stored pollen.

Paternity analysis

For the paternity analysis, leaf samples should be collected only after the plants have reached a minimum of 30 cm in height, after about 30 to 40 days. The effectiveness of the crossings with stored pollen can be proven by the molecular analysis of microsatellite markers (SSR). The paternity test recommended by AMARAL et al. (2012a; 2012B) includes three SSRs (SCC05, SCC06 and SCC92) previously studied for this purpose, in the genetic resource laboratory in the Campus of Arapiraca, of the Federal University of Alagoas. The SSR sequences are listed in Table one. In the profile analysis of microsatellites, the offsprings of each crossing need be classified as true hybrids, selfing or contaminants. Understanding the paternity analysis, the progeny which shows the presence of specific male alleles (stored pollen) must be classified as a true hybrid between both parents. The progeny which shows only female alleles must be classified as resulting from self-pollination. If the progeny shows distinct male alleles, the offspring must be considered as resulting from contaminated pollen or "aliens".

Table 1. The sequence of three microsatellite markers (SSR) applied for paternity test of sugarcane plants obtained with conserved pollen.

SSR	GenBank ID	Motif	Primer (5'- 3')
SCC05	CA205346	$GATA_{(10)}$	F: CGGAATCCAATTCGTACGTT R: CATTGGTTGCACCACAGTTC
SCC06	CA207738	$AAAG_{(10)}$	F: TATTCCACCGGGAACAAGAA R: GGGATTGTAGCGACGAGTTG
SCC92	CA210595	$ATCT_{(15)}$	F: CTCCGCATTAGCCATTTCC R: TGGTACTCGTCCATGTCGTC

Final Considerations

The methodology of stored pollen allowed to obtain plants from crossings between asynchronous varieties at Ridesa BAG-AL in 2009 and 2012.

The paternity analyses proved the efficiency of this storage method and highlighted the potential of the application in the sugarcane breeding program routine.

This method of storing pollen has a potential for performing interspecific crossings including in Saccharum complex for creating pollen banks; for setting up a new proposal for ex situ conservation and acquisition/exchange of genetic material (pollen) between sugarcane breeding programs; for importing/exporting pollen, for researching about pollen in places where there is no spontaneous sugarcane flowering; and for biotechnological applications, for example, when pollen grains are preserved, which will benefit the application of the genetic transformation.

Acknowledgements

Thanks for the efforts of Embrapa and Ridesa for establishing the partnership in research and financial support.

To the technicians at the Serra do Ouro Crossing and Flowering Station. A very special thanks to dedication of the technicals Edmundo Leobino da Silva and José Cicero Pereira.

To Professor Cicero Carlos Almeida for their support in molecular paternity analyzes.

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Ministry of Agriculture, Livestock and Food Supply

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1st edition (2013)

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Layout, graphic design and editing: *Ailla Freire de Azevedo*